Opiate Receptor Changes after Chronic Exposure to Agonists and Antagonists

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The responsiveness of neurotransmitter receptor systems is altered by exposure of the receptors to their agonist and antagonist ligands. For agonists, the physiological response declines over time despite the presence of a constant stimulus. Chronic exposure to antagonists, by contrast, can lead to an increase in the number of receptors, often accompanied by increased responsiveness to agonists.

Three major processes, characterized in greatest detail in the β-adrenergic system[1] but typical of many transmitter systems, have been postulated to contribute to the loss of responsiveness to agonists. These include (1) loss of coupling to G-proteins, (2) sequestration of receptors, and (3) down-regulation. Each of these mechanisms may contribute to different aspects of the loss of responsiveness, particularly with regard to the time course of the change. Thus, uncoupling occurs in seconds to minutes, sequestration in minutes, and down-regulation in minutes to hours. Phosphorylation events appear critical for some and may be involved in all of these processes.

For opiates and their receptors, the role of these processes in the changing responsiveness observed after exposure to opiates has remained elusive. As discussed in the concluding section, *in vivo* studies on changes in receptors in adult animals have yielded numerous, often conflicting results, including down-regulation, up-regulation, and no change. Studies in developing animals indicate that the perinatal period is a time when the animal is particularly sensitive to regulation of receptors by agonists. *In vitro* cell lines, however, have been particularly useful for studying changes in receptors and responsiveness after exposure to agonists. Homogeneity of cell type and receptor content as well as control of the kinetics of drug exposure are among the advantages of these preparations.

This report focuses primarily on recent findings in opiate-receptor–containing neuronal cell lines as models of cellular responses to chronic exposure to opiates and their antagonists. The conclusion will discuss implications of these cellular models for studies *in vivo*.

REGULATION OF MU RECEPTORS *IN VITRO* BY MORPHINE

Many aspects of opiate receptor regulation were characterized with cell lines containing delta receptors such as N4TG1[2] and NG108-15[3] cells. However, morphine and other compounds with clinical usefulness, as well as abuse potential, act primarily at the mu receptor, which is not present on these cells. Characterization of the mu receptor and its response to prolonged exposure to agonists *in vitro* is therefore of considerable importance. One of the first cell lines used in studying mu receptors was derived from pituitary cells. In these 7315c cells, Puttfarcken *et al.*[4,5] demonstrated that, as with delta sites in the NG108-15 cells,[3] two components of the response to morphine could be characterized: an early desensitization, or decline in the inhibitory effect of an acute exposure to morphine on cyclic AMP accumulation, followed at later times and higher doses by down-regulation of the mu receptors.

The first neuronal cell line in which mu receptor down-regulation by morphine was demonstrated[6,7] was the SH-SY5Y cell line, a subclone of SK-N-SH cells. When SH-SY5Y cells were exposed to morphine and the mu receptors were measured with the mu-selective ligand [^{3}H]-DAMGO (Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol), the maximum number of receptors (B_{max}) decreased over time. At about 3 h, the decrease was half-maximal. Maximal down-regulation occurred at about 24 h with little further change up to 72 h. The effect was reversible by naloxone and was dose-dependent, with half-maximal decreases occurring at a concentration of about 0.5 μM morphine. This dose corresponds well to the reported apparent affinity of morphine for the mu receptor in these culture conditions,[8] which is expected to be lower than that observed with isolated membranes. This difference is due to several factors including the reduction of agonist affinity by salts in the media and loss of agonist due to internalization.

The morphine-induced decrease in mu binding was also shown to be temperature-dependent, consistent with down-regulation processes in other systems. Muscarinic receptors present on SH-SY5Y cells were not affected by morphine, indicating that the down-regulation was homologous for opiate receptors.

Earlier studies with NG108-15 cells[3] had shown that efficacy of agonists correlated with the capacity to induce down-regulation of delta receptors and that partial agonists were ineffective at producing down-regulation. This idea was supported for mu receptors in SH-SY5Y cells because pentazocine, a partial agonist in SH-SY5Y cells,[9] failed to down-regulate the mu receptor.[7] Recent studies involving site-directed mutagenesis of adrenergic receptors[1] indicate that physical and functional receptor/G-protein coupling may not be identical, and that physical coupling may be more important for down-regulation. In general, however, efficacy should correlate with coupling, and thus with the capacity for down-regulation.

REGULATION BY MORPHINE OF MU AND DELTA RECEPTORS IN THE SAME CELLS

Although the SH-SY5Y cell line was initially used as a neuronal model for studying mu receptor responses to morphine, the cells also express delta receptors. The ratio of mu to delta receptors has been estimated to be from 2:1[10] to 5:1.[11,12] Under our culture conditions, the ratio of mu to delta sites is about 1.4 to 1.[13] Morphine binds preferentially to mu receptors, but it can also bind with lower affinity to delta receptors. We therefore tested whether morphine affected the delta sites that are coexpressed with mu sites in SH-SY5Y cells. The delta sites were also

down-regulated by morphine. This would not be expected from studies in NG108-15 cells, because morphine did not affect receptors in those cells, which contain only delta sites.[3] Furthermore, morphine down-regulated delta receptors to a greater extent than mu receptors in the SH-SY5Y cells. Thus, the regulation of delta sites was qualitatively different in NG108-15 and SH-SY5Y cells. One possibility is that morphine could be down-regulating delta sites by acting at the mu sites present in SH-SY5Y cells but not NG108-15 cells. Baumhaker *et al.*[14] suggested such a mechanism for the parent cell line, SK-N-SH, based on studies with the mu receptor alkylating agent β-funaltrexamine.

To test whether mu and delta sites could be separately regulated by morphine, we combined treatment with morphine and the mu antagonist CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2) or the delta antagonist ICI 174864 (N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH). CTAP reversed the effects of morphine on the mu (but not the delta) receptor and ICI 174864 reversed the down-regulation of the delta (but not the mu) receptor. The pharmacology of both antagonists, however, proved complex. CTAP alone at high doses down-regulated the delta receptor. This action was found to result from delta agonist activity because ICI 174864 could reverse the CTAP-induced down-regulation. CTAP could, therefore, have blocked morphine-induced mu receptor–mediated down-regulation of delta sites, and simultaneously caused down-regulation by its activity as a delta agonist. A dose (300 nM) was found, however, that completely antagonized morphine-induced down-regulation of mu receptors without down-regulating delta receptors by itself or blocking the morphine-induced down-regulation of delta receptors. Taken together with the reversal of morphine-induced down-regulation by the delta antagonist ICI 174864, these results indicate that activation of the mu receptor is not required, but activation of delta sites is required, for morphine to down-regulate the delta receptor in SH-SY5Y cells. These qualitative differences between NG108-15 and SH-SY5Y cells in the regulation of delta receptors may help elucidate critical mechanisms in their regulatory processes.

REGULATION OF MU AND DELTA SITES *IN VITRO* BY ANTAGONISTS

Shortly after establishment of opiate binding assays, it was shown that chronic administration of opiate antagonists up-regulated opiate receptors,[15] and the phenomenon has been well-characterized since then.[16–19] *In vitro* models of this process have not been as consistent: Barg *et al.*[20] found increased [^{3}H]-DADL binding in NG108-15 cells but Law *et al.*[3] found no effect on [^{3}H]-diprenorphine binding.

We found[7,13] that 24-h exposure to naloxone increased both mu and delta receptors by about 40% in SH-SY5Y cells, indicating that these cells provide a good model for antagonist effects on neuronal opiate receptors. Antagonists selective for receptor subtypes were also able to up-regulate their respective receptors, but the effects were not as robust as with naloxone. CTAP increased mu receptors by 20–30%. As described above, however, it also down-regulated delta sites at high doses as a result of its delta agonist activity.

The up-regulation by CTAP was observed even in the presence of high concentrations of morphine. This is consistent with studies of Yoburn *et al.*[18] showing that up-regulation is resistant to blockade by agonists and that less than full occupancy of the receptors is required for up-regulation.

Indirect mechanisms are generally thought to be involved in antagonist-induced up-regulation. Thus, a basal "tone" of agonist activity is thought to induce partial down-regulation that is "unmasked" in the presence of antagonist. In culture, the

source of this agonist activity could be the cells themselves or the media. SH-SY5Y cells are known to express both proenkephalin[21] and POMC.[22] In addition, we found that fetal calf serum contains material that can displace [^{3}H]-diprenorphine binding.[13] Several potential sources for "basal agonist activity" are therefore present in the cell culture and could be part of the mechanism of up-regulation by antagonists. Studies with the delta antagonist ICI 174,864, however, raised the additional possibility of a direct mechanism of antagonist-induced up-regulation.

ICI 174,864 up-regulated delta receptors in the SH-SY5Y cells, but unexpectedly it also up-regulated mu receptors.[13] It is not known to have mu antagonist properties,[23] but has been shown to exhibit "negative intrinsic activity" at the high-affinity GTPase associated with delta receptors in NG108-15 cells.[24] One of the earliest events in the signaling cascade for agonists acting at G-protein-coupled receptors is stimulation of GTPase. In NG108-15 cells, there is a low basal activation of GTPase even in the absence of agonist ligands, and ICI 174,864 can inhibit this activity. This inhibition, or "negative intrinsic activity," could be involved in a direct mechanism by which the antagonist up-regulates receptors. If this pathway is shared by mu and delta receptors, it could contribute to the combined up-regulation by ICI 174,864 of mu and delta sites in SH-SY5Y cells.

REGULATION OF RECEPTORS BY SELECTIVE AGONISTS

The use of agonists selective for mu or delta receptors permitted selective down-regulation of each of the sites.[13] PL017, an analogue of both β-casomorphin and morphiceptin that is one of the most selective mu agonists available, down-regulated mu sites in SH-SY5Y cells with an IC_{50} of 180 nM, but did not alter delta sites at concentrations up to 10,000 nM. The mu selective enkephalin analogue DAMGO also selectively down-regulated the receptors. Conversely, the highly selective delta agonist DPDPE down-regulated delta, but not mu receptors. DPDPE was effective at very low (sub-nanomolar) doses, indicating either high efficacy for the compound or relatively high sensitivity of the SH-SY5Y cells to delta receptor down-regulation.

REGULATION OF RECEPTORS BY DIFFERENTIATING AGENTS

One advantage of cell line models is that in the undifferentiated state the cells proliferate rapidly to permit generation of a sufficient population for experimentation. The cells may then be induced to differentiate into a number of morphologically and biochemically distinct phenotypes, some of which may include high concentrations of the receptor of interest. In SH-SY5Y cells, Yu *et al.*[11] showed that differentiation with retinoic acid (RA) increased mu receptors by 60%. We further characterized the effects of differentiating agents on opiate receptors[13] and found that delta receptors are also increased to about the same extent as mu receptors by RA, but differentiation with the phorbol agent TPA increased mu receptors without changing delta sites. Thus, the phenotypic ratio of mu to delta sites can be manipulated by the choice of differentiating agents.

IMPLICATIONS OF *IN VITRO* MODELS OF OPIATE RECEPTOR REGULATION FOR *IN VIVO* STUDIES AND FOR MODELS OF OPIATE TOLERANCE

Inasmuch as activation of a receptor is the first step in the signal transduction cascade, changes in receptor number or affinity provide an attractive mechanism to explain the loss of responsiveness that is characteristic of tolerance. It is most likely, however, that tolerance involves several molecular sites of action, including receptor, G-protein, and effector proteins as well as phosphorylation and transcriptional events. Measurable changes in the receptor number or affinity cannot fully account for the profile of changes that characterize tolerance. Indeed, tolerance can develop independently of down-regulation. Changes in any other single step in the signal cascade, however, are also unlikely by themselves to account for the full profile of tolerance. Further understanding of changes at each step is needed to develop a comprehensive understanding of the cellular mechanisms of tolerance.

Regulation by agonists of opiate receptors in brain has been highly variable. After *in vivo* chronic administration of agonists, opiate receptors in brain have been reported to decrease,[25,26] increase,[27–31] not change,[32,33] change in some, but not other brain areas,[34] and to change in amount and direction depending on the dose, efficacy, and selectivity of the agonist.[35,36]

The suggestion that efficacy is a critical factor in whether an agonist will induce down-regulation was first proposed on the basis of *in vitro* studies with the delta receptor[3] and was confirmed *in vitro* for the mu receptor.[7] Studies *in vivo* are consistent with this idea because the highly efficacious agonist etorphine decreased mu and delta receptors in rat brain[26] and mu receptors in mouse brain.[35] The high efficacy mu agonist DAMGO down-regulated mu receptors in SH-SY5Y cells[13] and in three of eight areas examined in rat brain.[34]

The dose of agonist required for down-regulation may also offer clues to differences in receptor regulation *in vivo* and *in vitro,* and perhaps between developing and adult animals. It has been suggested[35,37] that one reason that down-regulation is readily observed in culture but not *in vivo* is that the doses required are toxic *in vivo,* whereas cell cultures can handle higher doses. In the mu receptor-containing 7315c pituitary cells,[4,5] down-regulation required considerably higher doses and longer exposure times than did desensitization, which is the relatively rapid loss of the cyclicAMP response to the acute exposure to agonist. Desensitization and down-regulation were also found to be separable phenomena in earlier studies on the delta sites in NG108-15 cells.[3]

In the NG108-15 cells, however, low doses of highly efficacious compounds were capable of down-regulating receptors. In SH-SY5Y cells it has been reported that desensitization and down-regulation were not clearly distinguishable.[38] In addition, doses of morphine (IC_{50}) required for down-regulation of receptors in SH-SY5Y cells[7,13] were comparable to both the K_i for binding in culture and the doses required to induce the major functional response to morphine, which is inhibition of adenylate cyclase. Furthermore, the delta agonist DPDPE down-regulated the delta receptor at very low concentrations.[13] Thus, down-regulation does not necessarily require unusually high doses of agonists. Again, the efficacy of the agonist is an important consideration, and, for the *in vivo* situation, its pharmocokinetics and the relative sensitivity of systems mediating toxic effects of the drug may all contribute to differences observed *in vivo* and *in vitro.*

In the developing animal, related issues may contribute to the phenomenon

observed by us and others[39–42] that agonist-induced down-regulation is more readily apparent during the perinatal period than in later life. Less activity of metabolic enzymes[43] or a less developed blood-brain barrier could contribute to greater delivery of opiates to the brain in young animals. Other factors must also be considered, however, including differential ontogenic expression of opiate receptor types or components of their signaling processes, and homeostatic mechanisms (described below) that may counteract the effects of opiates.

Like all models of tolerance, those involving changes in receptors have certain strengths and weaknesses.[44] Changes in receptors would account for certain aspects of cross-tolerance, in which agonists selective for one receptor type do not induce tolerance at other receptors. Theoretical models must accommodate the observation that opiates can induce changes in responsiveness of great magnitude (e.g., 1000 fold).[45] Considerable loss of response can be explained by receptor models. However, changes resulting from loss of receptor number alone would tend to be abrupt rather than continuous as is typical of tolerance. The concept of "receptor reserve," in which only a small proportion of receptors need to be occupied for a maximal response, in general does not specify the locus of the reserve. Excess of receptors or components of their signaling systems could mediate the reserve. Because chronic exposure to agonist diminishes the reserve, the dose-response curve of the agonist is first shifted to the right (greater doses are required for a given effect), and then the maximal response declines when the reserve is lost and the receptor number declines. Thus, in a system in which the receptor number contributes substantially to the reserve, a loss in number would be reflected in a gradual loss in response. If post-receptor mechanisms are primarily responsible for the reserve, loss of receptor number would lead to an abrupt drop in responsiveness.

One of the major weaknesses of an opiate receptor model of tolerance is that many studies *in vivo* have reported that tolerance occurs in the absence of changes in receptors. For this reason, several alternative mechanisms have been postulated to explain tolerance, some of which can be classified as homeostatic. In these models, non-opiate processes are activated by opiates and counteract the actions of the opiates. These mechanisms include "antiopiate" or "opiate modulating" endogenous peptides such as NeuropeptideFF (NPFF)[46–48] and brain peptides related to Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2). NPFF provides a model of a peptide that does not bind to opiate receptors but is able to antagonize opiate effects, presumably through actions at its own receptor. The Tyr-MIF-1–like peptides, by contrast, bind to both the mu opiate receptor[49–51] and to non-opiate Tyr-MIF-1 sites.[52–54]

Homeostatic mechanisms are thought to be activated by opiates acting at their receptors to release the modulating peptide. Although this model can account for aspects of tolerance and withdrawal that other models cannot, a theoretical limitation is that the level of activation of the modulating peptide will be maximal with saturation of the opiate receptor; this would occur relatively early in the development of tolerance, and further tolerance through this mechanism would be limited.[44] Tyr-MIF-1, Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2)[50] and related peptides, by contrast, can bind to the opiate site and, particularly during tolerance or in conditions of reduced opiate receptor reserve, can antagonize the actions of morphine and DAMGO.[55] This effect may result from its action as a partial agonist at the opiate site, from an "antiopiate" action at its own site, or both. These peptides therefore provide candidate molecules involved in a model of tolerance that combines aspects of opiate receptor and homeostatic mechanisms.[56] Such a combined model could account for aspects of tolerance that other models have difficulty explaining, such as large, continuous shifts in the dose-response curve.

In summary, several mechanisms and characteristics of agonist- and antagonist-

induced changes in opiate receptors and their responsiveness to opiates have been characterized in cell lines, including the mu receptor-containing SH-SY5Y cells. These studies have implications for changes observed *in vivo* and ultimately for our understanding of the dynamic processes underlying changing responsiveness to opiates and neurotransmitters in general.

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